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## Conservation genetics of Ireland's sole population of the River water crowfoot (*Ranunculus fluitans* Lam.)

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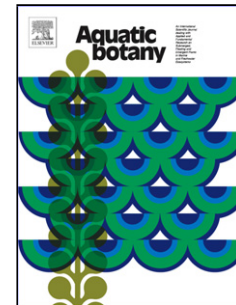
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**Highlights**

- We carried out genetic analysis on Ireland's sole population of *Ranunculus fluitans*
- Levels of genetic diversity were comparable to those in a more extensive population
- Very little evidence of hybridization with *Ranunculus penicillatus* was found
- No evidence of clonal reproduction was found
- Although genetically healthy, the population may be vulnerable to stochastic events

**Conservation genetics of Ireland's sole population of the River  
Water Crowfoot (*Ranunculus fluitans* Lam.)**

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## ABSTRACT

Populations of many freshwater species are becoming increasingly threatened as a result of a wide range of anthropogenically mediated factors. In the present study, we wanted to assess levels and patterns of genetic diversity in Ireland's sole population of the River water crowfoot (*Ranunculus fluitans*), which is restricted to a 12 km stretch of a single river, to assist the formation of conservation strategies. Analysis using amplified fragment length polymorphism (AFLP) indicated comparable levels of genetic diversity to those exhibited by a more extensive population of the species in England, and revealed no evidence of clonal reproduction. Allele-specific PCR analysis of five nuclear single nucleotide polymorphisms (SNPs) indicated no evidence of hybridization with its more abundant congener *R. penicillatus*, despite previous anecdotal reports of the occurrence of hybrids. Although the population currently exhibits healthy levels of genetic diversity and is not at risk of genetic assimilation via hybridization with *R. penicillatus*, it still remains vulnerable to other factors such as stochastic events and invasive species.

**Keywords:** AFLP, aquatic plants, hybridization, *Ranunculus*, SNPs, vegetative reproduction

## 1. Introduction

Populations of many freshwater species are becoming increasingly threatened as a result of a wide range of anthropogenically mediated factors, including geomorphic and hydrologic change such as drainage or the construction of dams, eutrophication, pollution, the introduction of invasive species and climate change (reviewed in Brinson and Malvárez 2002). Plants associated with rivers and lakes are particularly at risk due to their sessile nature and limited capacity for dispersal. In Great Britain and Ireland, many riverine and riparian plant species are now classed as endangered or threatened and several are the subject of Species Action Plans.

*Ranunculus fluitans* Lam. (River water crowfoot) is a subaquatic, perennial herb found in fast-flowing streams across western and central Europe, including the UK, as well as in southern Scandinavia. In Britain, the species is common in northern and central England, but is more sporadically found in southern England, Wales and southern and eastern Scotland (Perring & Walters 1976). In Ireland, the species is restricted to a single 12 km stretch of the Six Mile Water, Co. Antrim. It was first identified in 1865 and has been recorded only in the same section of the river since then (Beesley 2006). *R. fluitans* is monoecious, and subaquatic pollination occurs as a result of the formation of an air bubble in the perianth (Arber 1920), although it has been suggested that vegetative reproduction may be more common (Zander and Wiegand 1987; Cook 1990; Barrat-Segretain 1996). The species is occasionally found growing with *R. penicillatus* (Stream water crowfoot) and, although morphological differentiation between the two can be difficult, there are anecdotal reports of sterile hybrids between the species occurring in the sole Irish population (Hackney 1992).

The aim of the current project was to obtain genetic data necessary for the formulation of a rational, sustainable conservation programme for the remaining Irish population of *R.*

*fluitans*. Specifically, we wanted to determine whether extensive clonal growth had led to low levels of genetic diversity, which has been demonstrated previously in small, isolated plant populations (Beatty et al. 2008 and references therein), since low levels of genetic variation are generally correlated with decreased adaptive potential (Young et al. 1996). We also wanted to determine whether hybridization with *R. penicillatus* was occurring, since hybridization between rare species and a common congener can lead to introgression and even extinction of the rare species' gene pool via "genetic assimilation" (Levin et al. 1996; Rhymer and Simberloff 1996; Beatty et al. 2010).

## 2. Materials and methods

### 2.1. Surveys and sampling

Samples of *Ranunculus* spp. were collected along a 16km stretch of the main channel of the Six Mile Water River between Ballyclare and upstream of Antrim town, County Antrim during late summer 2008 (Figure 1). Samples were obtained by wading into the river channel where conditions allowed or by the use of a grapple in deeper water. A piece of *Ranunculus* spp. was removed from each individual plant encountered and placed in a labelled, sealed plastic bag. Subsequent molecular analysis (see Results) confirmed that each sample corresponded to a distinct plant, as opposed to ramets of the same clone. An accurate grid reference for the location of each plant was taken using GPS. Measurements of river water pH were taken at intervals of 1km along the stretch of river surveyed using a YSI 600 XLM multi-parameter meter. For comparison with the sole Irish population of *R. fluitans*, 30 individuals from the same species from the River Wye at Lower Bullingham, Herefordshire, were also sampled for analysis.

### 2.2. Molecular identification of *Ranunculus* species

Because it can be difficult to discriminate between aquatic *Ranunculus* species, molecular genetic techniques were used to clarify species identification. Species-specific single-nucleotide polymorphisms (SNPs) were identified by sequencing regions of the chloroplast genome amplified using universal primers (Grivet et al. 2001). A diagnostic SNP in the *atpH-atpI* intergenic spacer was screened using the allele-specific PCR (AS-PCR) technique described in Beatty et al. (2010) and the following primers: forward primer 5'-



ATAAGGACCTAGTTCTTGCATTTC-3'; reverse primer 5'-

ATAGTAGATATTTACTAGTTATATATGAAC-3'; *R. fluitans*-specific primer 5'-

CTGGTCCAAATGAGTAAACAGAG-3'.

### 2.3. Amplified fragment length polymorphism (AFLP) analysis

AFLP was carried out using the protocol of Vos et al. (1995). For each individual, 50 ng genomic DNA were digested using *EcoRI* and *MseI*. Following adaptor ligation and preamplification, 44 primer combinations were tested for clarity of amplification. Selective amplification was carried out using two primer combinations: E+AGC/M+CTT and E+AGC/M+CGCT. In each case, the *EcoRI* primer was fluorescently labeled with 6-FAM. Products were resolved on an AB3730xl capillary genotyping system and allele sizes were scored using LIZ-500 size standards. To estimate error rates, twelve samples (four *R. penicillatus* and four from each of the *R. fluitans* populations) were replicated from the digestion/ligation stage.

### 2.4. Single nucleotide polymorphism analysis of potential hybridisation in *R. fluitans*

Nuclear species-specific single nucleotide polymorphisms (SNPs) were ascertained using the method described in Beatty et al. (2010). 23 pairs of primers were designed from unique cloned fragments and used to amplify anonymous regions of the genome in an ascertainment set of *R. fluitans* and *R. penicillatus* individuals (primer sequences available on request). Allele-specific PCR (AS-PCR) primers were designed to screen five diagnostic SNPs that were fixed for alternate alleles in the two species (Table 1) and screened as described in Beatty et al. (2010). Both SNP alleles were screened at each locus to identify heterozygotes.

## 2.5. Data analysis

A Mann-Whitney U test was carried out to test for any correlation between the occurrence of the two species and water pH.

AFLP fragments were scored using the GENEMAPPER software package (V4.1; Applied Biosystems). Number of polymorphic loci ( $\#P$ ) and percentage of polymorphic loci ( $\%P$ ) with allele frequencies of between 0.05 and 0.95, and gene diversity ( $H_j$ ; Nei 1987; Lynch & Milligan 1994), calculated under the assumption of Hardy-Weinberg equilibrium, were estimated using the AFLP-SURV software package (V1.0; Vekemans 2002).

The potential occurrence of hybridization based on the AFLP data set was assessed using a Bayesian procedure implemented in the STRUCTURE software package (V2.3.3; Pritchard et al. 2000). The program was run using no prior knowledge and the admixture ancestry model. Five independent runs were carried out for each value of  $K$ , the number of genetic clusters, up to  $K = 5$ . Each Markov chain Monte Carlo analysis used a burn-in period of 10,000 followed by a further 100,000 iterations. The most likely value for  $K$  was estimated using the  $\Delta K$  statistic of Evanno et al. (2005) implemented in the STRUCTURE HARVESTER software package (V0.6.1; Earl et al. 2012).

To further investigate the genetic relationships between individuals, a principal coordinate analysis (PCOA) was carried out in GENALEX (V6.1; Peakall & Smouse 2006). Inter-individual genetic distances based on Nei & Li's (1979) distance measure were calculated from the AFLP data set using PAUP\* (V4.10). The PCOA was carried out using the standard covariance approach.

### 3. Results

Molecular species identification confirmed the morphological identification of all samples, since in each case the sample exhibited the expected *R. fluitans* or *R. penicillatus* SNP allele. In total, samples from 36 individual *R. fluitans* plants were collected from the Six Mile Water. With a single exception, all were restricted to the lower end of the Six Mile Water, downstream of Dunadry Bridge (Figure 1). All upstream individuals, with the exception of the single *R. fluitans* plant, were identified as *R. penicillatus*, and there was very little overlap between the two. Distribution of the two species was not correlated with pH, which was approximately constant along the length of the river (range 8.31 – 8.64), and which did not differ between the upstream and downstream sections (Mann-Whitney  $U = 5.00$ ,  $P = 0.136$ ).

The E+AGC/M+CTT and E+AGC/M+CGCT primer pairs amplified 222 and 224 polymorphic fragments respectively (446 in total), with an average reproducibility of 0.954. Diversity values (number of polymorphic loci, percentage of polymorphic loci and gene diversity) were broadly similar across all three populations studied (Table 2). Using the reproducibility value as a threshold, no potentially clonal individuals were identified i.e. those that differed by less than 4.6%.

The STRUCTURE analysis based on the AFLP data indicated that  $K = 2$  was the most likely number of genetic clusters, separating *R. penicillatus* from both *R. fluitans* populations, although there was some evidence of potential introgression in several individuals (Figure 2). The species-specific SNPs did not identify any hybrids between *R. fluitans* and *R. penicillatus*, but the PCOA based on the AFLP data, which clearly separated all three populations, did identify a single individual classified as *R. fluitans* that could potentially represent a hybrid (Figure 3).

#### 4. Discussion

*Ranunculus fluitans* in Ireland is restricted to the Six Mile Water downstream of Dunadry Bridge, Co. Antrim. It mainly grows separately from *R. penicillatus*, contrary to previous observations (Hackney 1992), with the only potential area of overlap now being free from aquatic *Ranunculus* species. It is possible that both occurred sympatrically in this area previously and that they have since been eradicated, possibly due to pollution events (Hackney 1992) or to dredging, although a previous ecological study on aquatic *Ranunculus* communities in France and Luxemburg also found that *R. fluitans* tends to occur separately from congeneric species (Mony et al. 2006). The main parameter separating *R. fluitans* communities from those of *R. penicillatus* and *R. peltatus* in the previous study was water alkalinity, but pH values in the present study were largely constant along the length of the river and not significantly different between upstream areas where *R. penicillatus* is found, and downstream areas where *R. fluitans* is found. A previous study suggesting that *R. fluitans* is primarily a eutrophic species (Trémolières et al. 1994) were not consistent with the findings of Mony et al. (2006), who noted that eutrophication presented a problem for Batrachian *Ranunculus* species in general in the UK. Likewise, they reported no difference in associated hydrophyte and helophyte species between *R. fluitans* and *R. penicillatus*.

Levels of genetic diversity in the Six Mile Water population of *R. fluitans* were similar to those found in the samples collected from the River Wye, where the species is far more abundant. Although most aquatic *Ranunculus* species have been shown to exhibit predominantly vegetative growth (Barrat-Segretain 1996), we detected no evidence of clonality. Many plant species possess the capacity for both sexual and asexual reproduction and, in many cases, the switch to asexual reproduction is associated with existence in sub-optimal conditions, where populations are small and/or fragmented (e.g. Honnay and Bossuyt

2005, Beatty et al. 2008). Although *R. fluitans* occupies an extremely restricted area in Ireland, regular flowering is observed in the population, and sexual reproduction would appear to predominate over vegetative propagation.

Previous studies have indicated that the high levels of vegetative reproduction observed in many populations of *Ranunculus* subgenus *Batrachium* species are associated with polyploidy and/or the formation of hybrids (Cook 1966; Holmes 1980; Dahlgren 1993; Barrat-Segretain 1996). Although hybrids between *R. fluitans* and *R. penicillatus* have been reported previously from the Six Mile Water (Hackney 1992), only a single potential case of hybridization was identified in the present study. As hybrids are sterile, and thus cannot contribute to successive generations (Barrat-Segretain 1996), these historical specimens may represent a transient episode of hybridization which is no longer ongoing to any great extent, given the current allopatric distributions of *R. fluitans* and *R. penicillatus* on the Six Mile Water. Nevertheless, the approximately 60%/40% assignment probability for the putative hybrid sample is close to that expected in a (sterile)  $F_1$  individual. Although the genome-wide AFLP analysis carried out in the present study can be used to identify putative hybrids, previous theoretical studies have shown that the five species-specific, codominant SNP markers used here are sufficient to differentiate between parental,  $F_1$ ,  $F_2$  and simple backcross classes (Vähä and Primmer 2006). Consequently, hybridization does not appear to represent a threat to the Irish population of *R. fluitans*, either by genetic assimilation via its more abundant congeneric species (Levin et al. 1996; Beatty et al. 2010), or by the increased incidence of vegetative reproduction associated with hybridization and consequent reduction in genetic diversity.

As the current levels of genetic diversity in the Irish population are comparable to those observed in the more extensive English population, it would appear that the restricted distribution of *R. fluitans* in Ireland has not yet negatively impacted genetic variation via the

processes of drift and/or inbreeding generally associated with isolated and fragmented plant populations (Young et al. 1996). Nevertheless, given its highly restricted distribution, the sole Irish population of *R. fluitans* is particularly vulnerable to extinction via stochastic effects including flooding, pollution and disease. Consequently, the population requires regular monitoring for conservation and management purposes. Herbivory by *Gammarus* spp. has also been shown to have a “devastating” effect on the species (Eichenberger and Weilenmann 1982) and thus the invasive *G. pulex*, which is spreading in Northern Ireland (Kelly et al. 2006), may also represent a threat to the species.

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**Table 1** *Ranunculus fluitans* / *R. penicillatus* AS-PCR primers

Locus	SNP	Flanking primers	SNP primers
RF103	A → T	GTTTTTTAATCAAATCAAAGCAACTTTTC	GAATTCGGTGCAATTACAAGAGGT
		GTTTAATTTCCACTTGTCGTTGG	GAATTCGGTGCAATTACAAGAGGA
RF110	11 bp indel	ACCCAATTCCCCAGAAAAAC	GCTCTTTGATTCAATTTCTGATTAG
		CACTCTGCTATCAAACCCTAATC	TTCTTTGATTGCGATTTCTGATTTCTG
RF216	T → C	CTCACCGCCACCACTC	AGTGGGAATTTTAGGAATGGCG
		ATCAAGGATTCAAACCGGAATGT	GGAGTTGGAATTTTATGAATGGCA
RF224	C → G	TCTTCGTAATTCCGTAAACCTCA	GTCGAAGGGTGGAGCTTATG
		TTCAACTTGTCAACAACCGATTCA	GTCGAAGGGTGGAGCTTATC
RF266	C → T	CCAAGTTATTACTTTGATAGATGGC	GTTACTATAAACAACTAAAACCATCAA
		CAATAACCTGGTCATCGATCTATG	CTAGAAACAACTATAACCCTCAG

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*R. penicillatus*-specific primer at top

**Table 2** Diversity statistics for the *Ranunculus* populations analyzed in the present study.  $N$  – number of individual plants sampled from each site.  $\#P$  – number of polymorphic loci;  $\%P$  – percentage of polymorphic loci;  $H_j$  – Gene diversity.

Species	Location	Lat (N)	Long (W)	$N$	$\#P$	$\%P$	$H_j \pm (SE)$
<i>R. fluitans</i>	Six Mile Water, Co. Antrim	54.7	6.1 – 6.2	36	155	34.8	0.126 (0.007)
	River Wye, Herefordshire	52.0	2.7	30	163	36.5	0.120 (0.008)
<i>R. penicillatus</i>	Six Mile Water, Co. Antrim	54.7	6.0 – 6.1	29	165	37.0	0.131 (0.008)

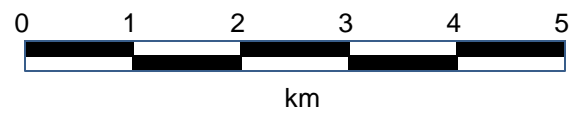
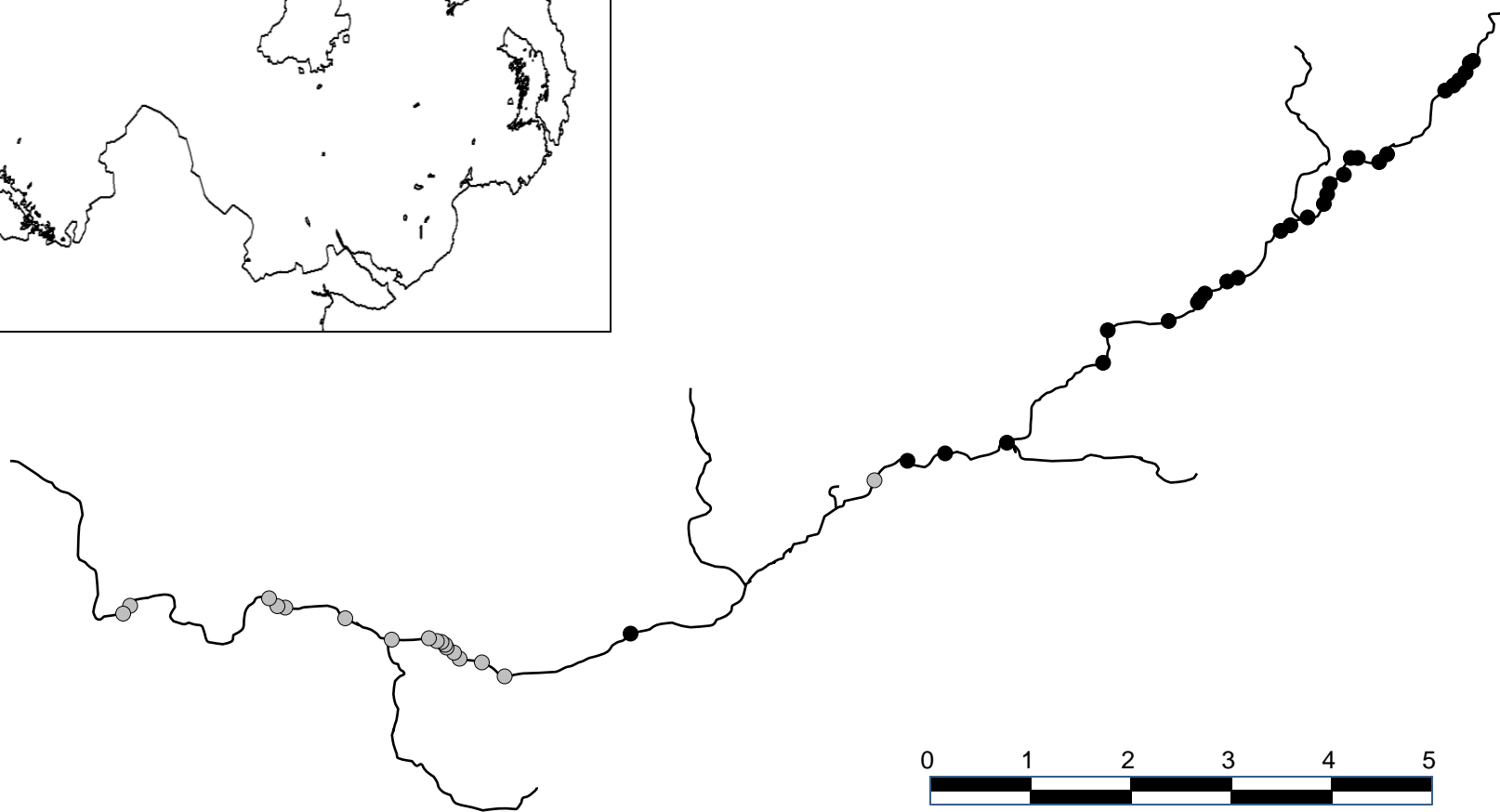
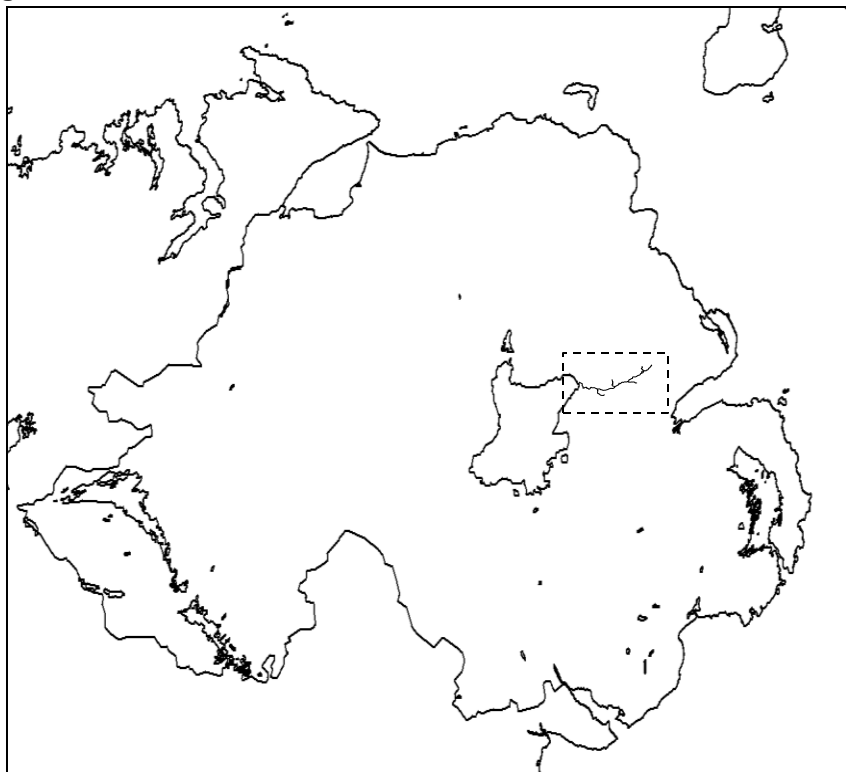
## Figure Legends

**Fig. 1.** Map showing the locations of samples used in the present study. Black circles represent *R. penicillatus* and grey circles represent *R. fluitans*. Inset map shows the location of the Six Mile Water in Northern Ireland.

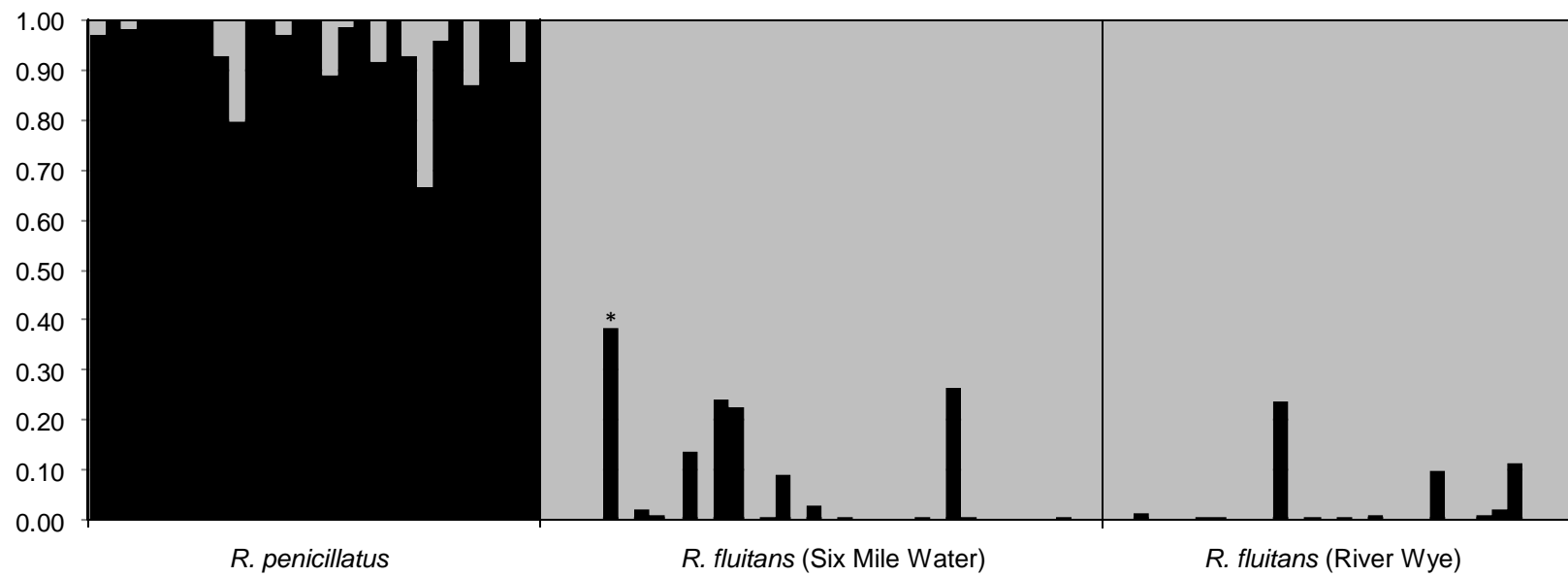
**Fig. 2.** Results of the Bayesian clustering analysis performed using STRUCTURE (V2.3.3). Each column represents an individual, with the height of each coloured segment indicating the probability of membership to each of  $K = 2$  genetic clusters. The individual marked with an asterisk corresponds to the potential hybrid individual highlighted in Figure 3.

**Fig. 3.** Principal component analysis showing relationships between individuals studied based on 446 AFLP markers.

Figure



## Figure



Figure

